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Impact of Methyl 5-*O*-(*E*)-Feruloyl-α-L-Arabinofuranoside on In-vitro Degradation of Cellulose and Xylan

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Abstract: The potential for inhibition of ruminal cell wall degradation by soluble phenolic acid–carbohydrate complexes was assessed using a synthetic molecule, methyl 5-*O*-(*E*)-feruloyl-α-L-arabinofuranoside (FA-Ara). In-vitro 24 and 72 h degradabilities of glucose from microcrystalline cellulose, and neutral sugars and uronic acids of oat spelts xylan were determined. Treatments included 0 (Control), 15, 150, 1500, and 4500 μmol liter^{-1} of methyl α-L-arabinofuranoside (Me-Ara), FA-Ara, or ferulic acid (FA). Additions of 1500 and 4500 μmol liter^{-1} Me-Ara; 15 and 150 μmol liter^{-1} FA-Ara; as well as 4500 μmol liter^{-1} FA significantly ($P < 0.05$) decreased 24 h cellulose degradation. Arabinose degradation from xylan after 24 h was decreased ($P < 0.05$) by 1500 and 4500 μmol liter^{-1} concentrations of Me-Ara and FA-Ara. No inhibitory effects of added compounds on 72 h glucose, xylose, or arabinose degradability were observed. Very little FA-Ara remained intact after 24 h. Following 72 h, no added FA-Ara was detectable, suggesting extensive degradation by microbial feruloyl esterases. Extent of polysaccharide degradation, *in vitro*, was not limited by a soluble phenolic acid–carbohydrate compound, which accurately models a known cell wall complex.

Key words: cellulose, xylan, cell wall, ferulic acid ester, arabinose, methyl 5-*O*-(*E*)-feruloyl-α-L-arabinofuranoside.

INTRODUCTION

Forage tissue contains phenolic acids, which may be present in various forms. *p*-Coumaric and ferulic acids constitute the major phenolic acid components in plant cell walls (Jung 1989). Phenolic acids exist in cell walls as ester-bound and ether-bound monomers, as esterified dimers, and as ester and ether bound cross-links between polysaccharide and lignin (Scalbert *et al* 1985; Hartley and Ford 1989; Iiyama *et al* 1990; Lam *et al* 1992). A toxic effect of phenolic cell wall components on the rumen microbiota is one possible mechanism that may explain constraints which cell wall lignification may impose upon fermentation of forage cell wall polysaccharides (Jung and Deetz 1993). Phenolic acid–carbohydrate complexes are released from cell walls during microbial cell wall degradation (Jung 1988) and are present in the rumen at concentrations ranging from 0·15 to 7·92 μmol liter^{-1} (Jung *et al* 1983a). Free phenolic acids are present in very low concentrations during in-vivo cell wall degradation (Jung *et al* 1983b). Concentrations of phenolic acids used in in-vitro experiments to investigate inhibition by phenolics of forage fiber

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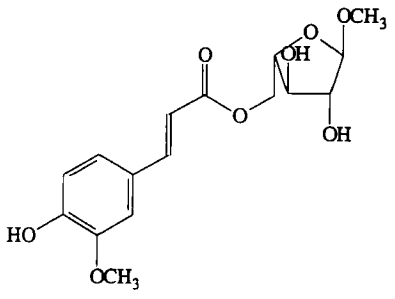

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degradation are often at least 10 times greater than levels observed *in vivo* (Chesson *et al* 1982).

Soluble phenolic acid–carbohydrate complexes consist mainly of *p*-coumaric or ferulic acid esterified to cell wall fragments, usually arabinose–xylose dimers or arabinose–xylose–xylose trimers (Gaillard and Richards 1975; Conchie *et al* 1988). Experiments with soluble phenolic acid–carbohydrate complexes, rather than free phenolic acids, may be more germane to the study of toxic relationships between phenolic compounds and cell wall polysaccharide degradation. Synthetic methyl-esters and butyl-esters of *p*-coumaric acid were more inhibitory to in-vitro forage fiber degradation than free *p*-coumaric acid when included in fermentations (Hartley and Akin 1989).

Methyl-5-*O*-(*E*)-feruloyl-α-L-arabinofuranoside (**I**) is a realistic and accurate model of complexes released during ruminal fermentation of forage cell walls (Hatfield *et al* 1991). The objective of this experiment was to assess potential for inhibition of in-vitro degradation of cellulose and xylan components by this soluble phenolic acid–carbohydrate complex.

EXPERIMENTAL

Methyl α-L-arabinofuranoside (Me-Ara) and methyl 5-*O*-(*E*)-feruloyl-α-L-arabinofuranoside (FA-Ara) were synthesized according to Helm *et al* (1992). Compounds were dissolved in water and lyophilized in order to liberate methylene chloride remaining after synthesis and crystallization. Stock solutions were prepared by dissolving (Me-Ara, FA-Ara) or suspending (FA) compounds in water, and were serially diluted to desired concentrations prior to addition to in-vitro fermentation vessels.

Microcrystalline cellulose (Sigma Chemical Company, St Louis, MO, USA) or oat spelts xylan (Sigma) (100 mg) were placed in 15 ml screw-cap centrifuge tubes and inoculated with 10 ml of diluted ruminal fluid (200 ml liter^{-1} strained rumen fluid: 800 ml liter^{-1} McDougall's buffer). Rumen fluid was obtained from a nonlactating Holstein cow (*Bos taurus* L) fed grass hay. Equimolar initial concentrations of added compounds were achieved by adding 1 ml of appropriately diluted stock solutions to 10 ml in-vitro batch culture fermentations. Concentrations of Me-Ara, FA-Ara and FA used in this experiment included: 0 (control; 1 ml water), 15, 150, 1500 and 4500 μmol liter^{-1}. Substrates were fermented for 24 and 72 h at 39°C. Fermentation vessels were mixed twice daily during incubation. Three replicate fermentation vessels were included for each combination of substrate, treatment and time variable. Following fermentation, 1 ml of supernatant was withdrawn and frozen for estimation of the extent of FA-Ara degradation. Culture supernatant pH was recorded. Contents of batch culture vessels were washed with water into 50 ml screw-cap centrifuge vials and lyophilized.

Neutral sugar content of substrates and undigested residues were determined by two-stage acid hydrolysis (Theander and Westerlund 1986) and were quantified by high-performance liquid chromatography (Jung and Russelle 1991). During hydrolysis, an aliquot (1 ml) was reserved for estimation of uronic acids in oat spelts xylan (Ahmed and Labavitch 1977), using galacturonic acid as the reference standard. Klason lignin content of oat spelts xylan was determined as the ash-corrected hydrolysis residue (Theander and Westerlund 1986). Degradability of cell wall neutral sugars and uronic acids in the substrates was calculated.

Amount of FA-Ara remaining following in-vitro fermentation was determined for diluted aliquots by measuring absorbance at 375 nm, by a method adapted from Hatfield *et al* (1991).

Design of the experiment was a split plot arrangement of treatments in a randomized complete block. Data were blocked by replicate. The statistical model contained added compound as the whole plot effect. Sub-plot effects were level of addition and the interaction of compound and level. Statistical analyses of data were accomplished using the general linear models procedure of SAS (SAS 1985). Differences between means for the control and individual compound–level combinations were tested by the least significant difference procedure of SAS when a significant ($P < 0.05$) compound × level interaction was observed in the analysis of variance.

RESULTS AND DISCUSSION

Oat spelts xylan used in this experiment contained xylose (632 g kg^{-1}), glucose (208 g kg^{-1}) Klason lignin (62 g kg^{-1}), arabinose (66 g kg^{-1}), galactose (5 g kg^{-1}) and uronic acids (20 g kg^{-1}) constituents. Microcrystalline cellulose yielded only glucose residues upon hydrolysis.

Presence of Me-Ara in the fermentation media at initial concentrations of 1500 and 4500 μmol liter^{-1} decreased ($P < 0.05$) 24 h cellulose degradability compared to the control (Table 1). The possible significance of this observation will be discussed later with regard to arabinose degradability. Inhibition ($P < 0.05$) of 24 h

TABLE 1
Degradability of glucose from microcrystalline cellulose

Compound	*Level* ($\mu mol\ liter^{-1}$)	*Glucose* ($g\ kg^{-1}$)	
		24 h	*72 h*
Control	0	$376^{c,d}$	852
Me-Ara	15	$361^{b,c,d}$	919
	150	$332^{a,b,c}$	847
	1500	$310^{a,b}$	832
	4500	$320^{a,b}$	836
FA-Ara	15	$318^{a,b}$	823
	150	307^{a}	871
	1500	389^{d}	827
	4500	380^{d}	833
FA	15	381^{d}	870
	150	$350^{a,b,c,d}$	930
	1500	$345^{a,b,c,d}$	912
	4500	$326^{a,b}$	845
SEM		15	27

[a–d] Means in the same column not sharing a letter differ significantly ($P < 0.05$).

cellulose degradability by addition of FA-Ara compared to the control was noted at the lower concentrations (15 and 150 μmol liter^{-1}). This inhibition was not noted, however, at greater concentrations of FA-Ara. Degradability of cellulose following 24 h was significantly depressed ($P < 0.05$) at the greatest FA concentration compared to the control. This level (4500 μmol liter^{-1}) is similar to the lowest levels used in other experiments where inhibitory influences of simple phenolic acids have been observed (Chesson *et al* 1982; Jung and Fahey 1983). Presence of ferulic acid at a 4500 μmol liter^{-1} concentration represents a level at least 500 times greater than levels of phenolic–carbohydrate complexes (0·15–7·92 μmol liter^{-1}) observed *in vivo* during the fermentation process (Jung *et al* 1983a).

In contrast, 24 h degradability of glucose residues from oat spelts xylan (Table 2), though more extensive than cellulose degradation, was unaffected by addition of the compounds.

Degradability of arabinose from xylan (Table 2) following 24 h of fermentation was significantly depressed by addition of 1500 and 4500 μmol liter^{-1} Me-Ara and FA-Ara compared to the control. The magnitude of this depression was unexpected. At both concentrations (1500 and 4500 μmol liter^{-1}), depression by the phenolic acid:carbohydrate complex was similar to or less than ($P > 0.05$) the depression elicited by addition of the methylated sugar analog to fermentations. Depression of 24 h arabinose degradability, as well as perhaps the transient depression of 24 h glucose (Table 1) and uronic acid degradability (Table 2) may represent a substrate affinity by rumen bacteria for soluble Me-Ara added to fermentations in preference to the isolated cell wall polysaccharides used as fermentation substrates in this experiment. Russell and Baldwin (1978) outlined a similar substrate affinity phenomenon and catabolite regulatory mechanisms for various species of rumen bacteria. In their report, soluble mono- and disaccharides (ie glucose and sucrose) added to incubations of *Selenomonas rurminantium* halted degra-

TABLE 2
Degradability of oat spelts xylan polysaccharide components

Compound	*Level* ($\mu mol\ liter^{-1}$)	*Xylose* ($g\ kg^{-1}$)		*Glucose* ($g\ kg^{-1}$)		*Arabinose* ($g\ kg^{-1}$)		*Uronic acids* ($g\ kg^{-1}$)	
		24 h	*72 h*	*24 h*	*72 h*	*24 h*	*72 h*	*24 h*	*72 h*
Control	0	296	717	745	981	$513^{d,e}$	846	548^{d}	751
Me-Ara	15	285	724	762	976	$505^{d,e}$	854	$520^{c,d}$	700
	150	229	712	723	964	469^{d}	857	$399^{a,b}$	735
	1500	273	716	726	981	309^{c}	850	$455^{a,b,c,d}$	748
	4500	341	672	769	980	16^{a}	792	$477^{b,c,d}$	730
FA-Ara	15	330	679	758	979	$546^{e,f}$	841	367^{a}	683
	150	297	778	730	978	$493^{d,e}$	871	$404^{a,b}$	676
	1500	306	740	720	986	374^{c}	854	$467^{b,c,d}$	734
	4500	415	661	767	978	182^{b}	811	$429^{a,b,c}$	670
FA	15	377	745	796	983	$604^{f,g}$	854	$379^{a,b}$	686
	150	365	775	816	971	$601^{f,g}$	871	$510^{c,d}$	729
	1500	362	823	794	966	$547^{e,f}$	888	$520^{c,d}$	714
	4500	527	795	823	964	653^{g}	855	$520^{c,d}$	708
SEM		22	32	18	6	23	21	33	28

[a–f] Means in the same column not sharing a letter differ significantly ($P < 0.05$).

dation of cellobiose until utilization of soluble added components was complete. These researchers did not use arabinose as a source of soluble monosaccharide in their work, however, a similar sequential utilization of carbohydrates may represent a plausible explanation for the magnitude of depression in degradability observed following 24 h incubation in our experiment. It is not known *if* Me-Ara, the methyl α-furanoside of L-arabinose, is metabolized by rumen bacteria in the same fashion as α-L-arabinofuranosyl side chains of forage arabinoxylans. Since the pH of 24 h fermentation supernatants was unaffected by addition of compounds in this experiment (data not shown), a negative influence of low pH on degradation of fiber components can be discounted as a cause of this depression in polysaccharide degradation.

Addition of FA apparently stimulated degradation of arabinose during the initial 24 h of fermentation. Stimulation of in-vitro hemicellulose fermentation by addition of phenolic acids has been previously reported (Jung 1985). The apparent stimulation observed in our experiment may reflect a requirement by *Ruminococcus albus* for 3-phenylpropanoic acid (Hungate and Stack 1982). Since 3-phenylpropanoic acid is a primary intermediate of anaerobic phenolic acid metabolism (Colberg and Young 1985), addition of FA to in-vitro fermentation vessels can provide a supplemental source of 3-phenylpropanoic acid to rumen microorganisms.

Degradability of uronic acids from oat spelts xylan following 24 h (Table 2) was decreased ($P < 0.05$) with the presence of 15, 150, and 4500 μmol liter^{-1} FA-Ara compared to the control. Response of 24 h uronic acid degradability to Me-Ara and FA was sporadic and inconsistent. Significant inhibition ($P < 0.05$) of initial uronic acid degradability compared to the control was only observed at relatively low concentrations of Me-Ara (150 μmol liter^{-1}) and FA (15 μmol liter^{-1}).

Following 72 h, degradability of glucose was extensive, averaging 860 g kg^{-1} for cellulose (Table 1) and 977 g kg^{-1} for glycosyl residues of oat spelts xylan (Table 2). Degradability after 72 h for glucose from cellulose and oat spelts xylan, as well as xylose, arabinose and uronic acids from oat spelts xylan (Table 2), was similar for all compounds tested and levels of addition. Any negative influence of added Me-Ara or FA-Ara on arabinose degradability was absent following 72 h. Supernatant pH after 72 h fermentation was not affected by addition of compounds (data not shown).

Additions of FA-Ara were detectable in diluted ruminal fluid. With initial concentrations of 4500 μmol liter^{-1} FA-Ara, only 6·9% remained intact following fermentation for 24 h. At initial concentrations less than 4500 μmol liter^{-1}, no intact FA-Ara was detectable in fermentation supernatants following 24 h. After 72 h, intact FA-Ara was absent from the fermentation supernatants at all levels of addition. The rapid and extensive disappearance of FA-Ara during fermentation suggests thorough action of feruloyl esterase enzymes. Presence of specific feruloyl esterase enzyme systems has been documented in *Fibrobacter succinogenes* (McDermid *et al* 1990) and various anaerobic fungal species (Borneman *et al* 1990). Synthetic FA-Ara is a suitable substrate for feruloyl esterase enzymes (Hatfield *et al* 1991).

CONCLUSIONS

We conclude from these results that extent of in-vitro polysaccharide degradation, reflected by 72 h degradability data from this experiment, was not limited by addition of a soluble phenolic acid:carbohydrate complex or FA. Depression of 24 h arabinose, glucose, and uronic acid degradability by the highest concentrations of FA-Ara was not greater than depressions elicited by equimolar concentrations of Me-Ara. Sizable depressions in arabinose degradability associated with the highest concentrations of Me-Ara are likely a result of expression by microorganisms of substrate affinity or of a catabolite regulatory paradigm. Feruloyl esterases were capable of completely degrading the synthetic compound, FA-Ara. We consider that ferulic acid and FA-Ara do not exert any appreciable negative or toxic influence on forage cell wall polysaccharide degradation *in vivo*.

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